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Comparison of UV and tandem mass spectrometric detection for the high-performance liquid chromatographic determination of diclofenac in microdialysis samples

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Abstract

High-performance liquid chromatography (HPLC) was used to analyze microdialysis samples obtained in vivo from human subcutaneous adipose tissue after topical application of the nonsteroidal anti-inflammatory drug diclofenac. For the reliable determination of diclofenac two different detection principles were applied in two different laboratories. One HPLC method utilized UV-detection at 280 nm, the other one used selected reaction monitoring mass spectrometry (MS). The HPLC-UV and -MS methods offered low limits of quantification of 10 and 1 ng/ml and an accuracy between 94.0–126.7 and 89.3–110.9%, respectively. However, a comparison showed that the HPLC-UV method failed to determine diclofenac in biological matrices, as both false negative and positive values were found. HPLC-MS is clearly superior to HPLC-UV due to a much more selective detection, increased sensitivity and shorter run times.

Keywords: Diclofenac; Microdialysis; Reversed-phase HPLC; UV detection; Tandem mass spectrometry

1. Introduction

Diclofenac (2-[(2,6-dichlorophenyl)amino] phenyl acetate) is a nonsteroidal anti-inflammatory drug of the phenylacetic acid class. As a potent inhibitor of the prostaglandin synthesis it has antipyretic, analgesic and anti-inflammatory activities [1,2]. Its chemical properties include a pK_a of 4.0, high solubility in ethanol, a very high plasma protein binding of 99.5%, and a half-life in plasma of 1–2 h [2].

The topical application of diclofenac in the treatment of localized inflammations like periarthritis and myogelosis has the advantage to achieve therapeutic effect without the risk of

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serious side effects that may occur after peroral administration [3]. However, contradictory findings have been reported on the actual penetration depth and locally attained concentrations after topical application [4–7]. In vivo microdialysis (MD) has been used to study the transdermal penetration of diclofenac after topical application [6,7]. MD is based on sampling of analytes from the interstitial space fluid by means of a semipermeable membrane. The major characteristics of MD samples are: (i) their lack of proteins; (ii) small sample volumes of 2–100 μl; (iii) low analyte concentrations; (iv) aqueous medium; and (v) high ionic (isotonic) strength [8]. Unfortunately, the small sample volumes preclude the use of preconcentration methods such as liquid–liquid extraction or solid phase extraction prior to analysis [9]. Today, MD is primarily limited by the availability of sufficiently sensitive assays [10]. Further requirements for the analysis of MD samples from clinical studies are that the assay must be able to handle with small sample volumes and a great number of samples.

Several analytical assays have been published so far for the quantification of diclofenac in different matrices such as plasma, urine, and human aqueous humor using high-performance liquid chromatography (HPLC)-UV [11–15], HPLC-electrochemical detection [16], gas chromatography–mass spectrometry (MS) [17,18], HPLC-MS [19,20], and capillary electrophoresis [20–22]. However, none of them was applied to small-volume MD samples with low analyte concentrations. Therefore, we developed two different methods to determine diclofenac in MD samples. Both methods, HPLC-UV and HPLC-tandem mass spectrometry were compared in regard to sensitivity, selectivity, accuracy, precision, and their suitability for the analysis of biological samples.

2. Experimental

2.1. Microdialysis

A commercially available MD probe CMA-10 (CMA, Stockholm, Sweden) with a molecular cut-off of 20 kDa, an outer diameter of 500 μm, and a membrane length of 16 mm was used. As previously described [9,10], the MD probes were inserted into the subcutaneous adipose tissue of the thigh of healthy volunteers. Voltaren® Emulgel® 1% (Novartis Pharma, Vienna, Austria) was applied to the surface of the thigh. The following perfusing media were used: (i) Ringer's solution (154 mM sodium chloride, 2.74 calcium chloride, and 4.02 mM potassium chloride, obtained from Mayrhofer Pharmazeutika GmbH, Linz, Austria), thereinafter called Ringer-MD; (ii) 3.3% serum albumin in Ringer's solution (prepared by diluting 5% human albumin obtained from Baxter, Vienna, Austria), thereinafter called albumin-MD. A flow rate of 1.5 μl/min was established by employing a microinfusion pump (Precidor; Infors, Basel, Switzerland). The samples were collected in 60 or 120 min intervals resulting in 90–180 μl sample volumes. The samples were split into equal portions and were stored at −80 °C prior to analysis by HPLC-UV or -MS.

2.2. HPLC-UV analysis

2.2.1. Instrumentation

The System Gold HPLC instrument consisted of a 508 autosampler and a 126 solvent delivery module (Beckman Coulter Inc., Fullerton, CA, USA). Ultraviolet detection was performed at 280 nm with a UV-975 detector (Japan Spectrometric Co., Osaka, Japan). Control of the HPLC system and data acquisition were performed with 32 Karat software (Beckman Coulter Inc.). The sample tray was cooled to 8 °C. Fifty microliters aliquots of the samples were injected onto the reversed-phase column Luna C8(2) (100 × 2.0 mm, 3 μm; Phenomenex, Torrance, CA, USA) that was maintained at 30 °C. The binary gradient utilized acetonitrile–water–acetic acid–triethylamine (200:791.18:8.17:0.65, v/v/v/v, pH* 3.6) as mobile phase A, and acetonitrile–water–acetic acid–triethylamine (600:391.18:8.17:0.65, v/v/v/v, pH* 4.5) as mobile phase B. The separation was initiated at a constant flow of 0.300 ml/min with 25% of B for 5 min, followed by an increase in B to 76.15% within 1 min. This % B was maintained for the next 15 min and then returned to the original

25% within 2 min. Each run lasted 23 min, with 2.5 min equilibration after each run.

2.2.2. Sample preparation

After thawing, Ringer-MD samples were directly injected onto the column. Samples with too small volumes were diluted with Ringer's solution to obtain a volume of 80 μl that was required for injection. Serum albumin-containing MD samples were deproteinized by adding twice the sample volume of methanol. After centrifugation at $5000 \times g$, 15 min, 4 °C, the supernatant was injected onto the HPLC column.

2.2.3. Quantification

Diclofenac sodium salt (Sigma, St. Louis, MO, USA) was dissolved in methanol and diluted with Ringer's solution to a concentration of 5000 ng/ml of the free acid. This stock solution stored at −20 °C was stable at least for 1 month. Fresh calibration solutions were prepared every day by diluting the 5000 ng/ml stock solution with Ringer's solution in the range of 1.2–1250 ng/ml. Quantification was achieved with an external calibration curve using the peak areas. For Ringer-MD samples, the calibration curve was established with diclofenac standards in Ringer's solution. For albumin-MD samples the calibration curve was recorded with spiked albumin solution within a concentration range of 4–500 ng/ml. For comparison purposes calibration standards were also prepared with drug-free MD samples spiked with diclofenac in the range of 1–16 ng/ml. Within- and between-day accuracy and precision of the assay were determined by analyzing diclofenac standards in Ringer's solution at concentrations of 20, 100, and 500 ng/ml in triplicate on 3 different days. The limit of detection (LOD) and limit of quantification (LOQ) was given as determined by signal-to-noise ratio of 3 and 10, respectively.

2.3. HPLC-tandem MS analysis

2.3.1. Instrumentation

LC–MS/MS analyses were performed on an API 3000 triple quadrupole mass spectrometer equipped with a nebulizer ion source which was operated at 500 °C in the positive ion mode (Sciex Instruments, Thornhill, Canada). This system was connected to a Series 200 autosampler and a micropump (Perkin Elmer, Wellesley, MA, USA). Both, the HPLC and MS/MS system were controlled with the Analyst Software (Applied Biosystems, Foster City, CA, USA). For selected reaction monitoring the following mass transitions were chosen: m/z 296.2 → 215.1 for diclofenac and 358.2 → 173.8 for the internal standard. A mixture of acetonitrile and 20 mM formic acid (53:47, v/v, pH 2–3) was used as mobile phase. Chromatographic separation was achieved on a Superspher RP select B column (125 × 3.0 mm, 5 μm; Merck, Darmstadt, Germany) at 40 °C. Each run lasted 5 min, with approximately 1 min equilibration time between two runs.

2.3.2. Sample preparation

After thawing, 25 μl of Ringer- or albumin-MD samples were mixed with 25 μl of a solution of the internal standard indomethacine (Fluka, Buchs, Switzerland) in methanol and 25 mM formic acid (1:1, v/v) resulting in a final concentration of 250 ng/ml. Twenty microliters aliquots of these samples were directly injected onto the HPLC-MS system.

2.3.3. Quantification

Calibration solutions in the range of 1.02–1047.1 ng/ml were prepared by diluting a 477 μg/ml stock solution with Ringer's solution. The resulting standards were stored at −30 °C prior to use. The linear regression parameters were determined by using the least-square-fit method with the peak area ratio of diclofenac and the internal standard versus the concentration. The factor 1/concentration was used as statistical weighting factor. Since the response of the mass spectrometer was not strictly linear, a coordinate transfer was performed according to $y \Rightarrow y^A$ ($0.98 \leq A \leq 1.01$).

Within- and between-day accuracy and precision of the assay were determined by analyzing three batches of diclofenac samples in Ringer's solution at concentrations of 2.70, 24.2, and 901.6 ng/ml. Each batch comprised five samples at each concentration level. The LOQ was estimated as the

lowest concentration, where a precision of below 20% could be achieved.

3. Results and discussion

Previous studies focussed on the topical application of diclofenac showed both high variability and very low levels of tissue concentration [6,7]. The minimal effective concentration of diclofenac in synovial fluid was estimated to be 100–500 ng/ml [1]. Therefore, tissue concentrations under 5 ng/ml seem to be clinically not relevant. Nevertheless, two different methods with very low sensitivity using either UV or MS detection were developed in two independent laboratories.

3.1. HPLC-UV analysis

HPLC-UV detection was used for the determination of diclofenac as this technique offers high sensitivity and as the instrumentation is easily accessible. Several HPLC-assays have been published for the determination of diclofenac in various matrices (see Ref. [1]). For example, Riegel and Ellis investigated successfully small volumes of ocular fluids at low diclofenac concentration levels [11]. Applying this method to MD-samples, diclofenac eluted near a large peak stemming from the matrix reducing the sensitivity of the assay. To get rid of the matrix components, several binary gradients and step-gradients with different buffers were tested. Finally, a step gradient was found that guaranteed an excellent sensitivity, since a smooth baseline was achieved. The injection volume was increased to 50 μl to obtain a better sensitivity. A partial HPLC-UV chromatogram of a blank Ringer-MD sample is given in Fig. 1a. A comparison to diclofenac standard in Ringer's solution with a concentration near the LOQ (Fig. 1b) shows that no interfering peaks elute near the diclofenac signal. A chromatogram of a Ringer-MD sample obtained from a human volunteer after topical application of diclofenac proves the detection limit in the low ng/ml level (Fig. 1c).

It is evident from the chromatograms of an albumin blank, a diclofenac spiked albumin sample, and an albumin-MD sample obtained from a volunteer after topical application of diclofenac (Fig. 2a–c) that none of the matrix peaks interfere with the analyte signal. The LOD and LOQ were higher as for the Ringer-MD samples, because the albumin-MD samples were diluted with organic solvent during the protein precipitation step. The retention time of diclofenac was found to be slightly reduced due to the increased methanol content of the injection solution.

Data concerning the performance of the assay are summarized in Table 1. Although the calibration curve was linear over the full concentration range (1.2–1250 ng/ml; correlation coefficient $R > 0.9996$), two different calibration lines had to be used for quantification of the MD samples. The slope of the calibration line was lower in the lower concentration range ($R > 0.993$) than in the upper concentration range ($R > 0.9996$) (see Table 1). This adaptation was necessary to achieve sufficient data accuracy for diclofenac levels near the LOQ. To determine the within- and between-day accuracy and precision of the method diclofenac standards in Ringer's solution were analyzed in triplicate on three different days, (see Table 2). The accuracy ranged from 94.0 to 126.7% with a precision better 6.0% R.S.D.

Generally, calibration curves should be generated in a matrix equal or at least similar to the sample matrix. Therefore, drug-free MD samples should be used for the preparation of calibration standards. Here, standard solutions of both diclofenac in Ringer's solution and spiked Ringer-MD samples were used to generate calibration curves to confirm the high sensitivity obtained. Slopes of both calibration curves were found in close agreement for the range between 1.2 and 20 ng/ml proving that there are no interfering components in the matrix. Therefore, in this case it is justifiable to use diclofenac standards in Ringer's solution for the quantification of Ringer-MD samples.

3.2. HPLC-tandem MS

Due to the small samples volumes associated with low analyte concentrations, an HPLC-tandem MS method was additionally developed with a required LOQ of 1 ng/ml. The molecular mass of the protonated molecular ion and the fragment

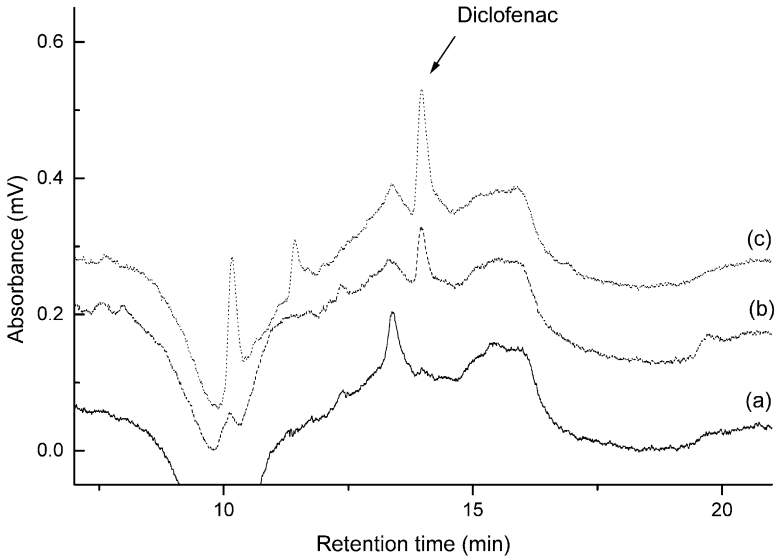

Fig. 1. Partial HPLC-UV chromatograms of: (a) a drug-free Ringer-MD sample; (b) diclofenac standard in Ringer's solution (concentration 7.8 ng/ml); (c) a Ringer-MD sample obtained in vivo from subcutaneous adipose tissue of a volunteer (calculated concentration 14.6 ng/ml).

with the highest abundance were selected as parent and product ions, respectively, for selective reaction monitoring. A comparison of the diclofenac selective reaction monitoring HPLC-MS chromatograms demonstrates the high specifity and sensitivity of the LC–MS method. Despite the

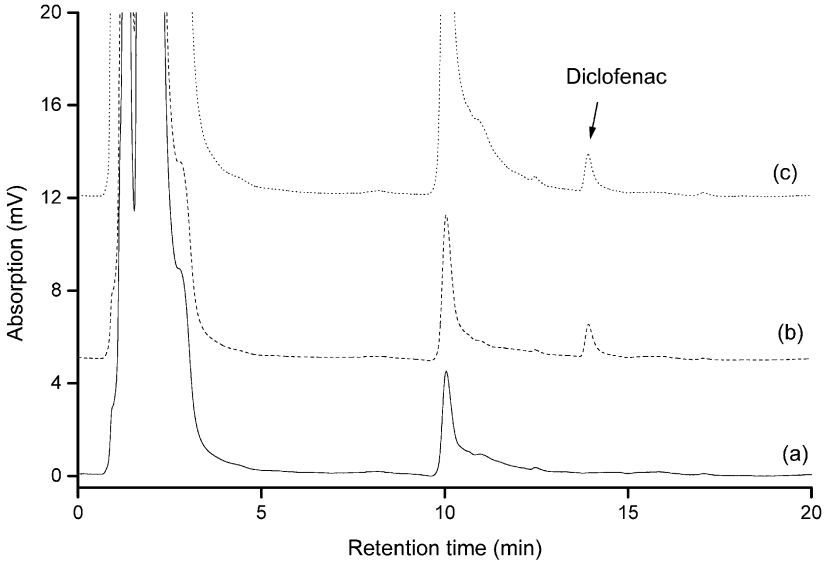

Fig. 2. HPLC-UV chromatograms of: (a) a blank serum albumin solution (3.3%); (b) diclofenac in 3.3% serum albumin solution (250 ng/ml); (c) an albumin-MD sample obtained in vivo from the subcutaneous adipose tissue of a volunteer (calculated concentration 266 ng/ml). The albumin-MD samples were extracted with methanol before injection.

Table 1
Performance of the HPLC-UV assay for the determination of diclofenac in Ringer's solution and in spiked serum albumin samples

	Diclofenac standard in Ringer's solution	Diclofenac spiked serum albumin (3.3%)
Retention time (min)	14.010±0.013	13.978±0.009
Range of calibration curve (ng/ml)	1.2–1250	4.0–500
Slope		
Low range (1.2–20 ng/ml)	178±21	87±5
High range (20–1250 ng/ml)	271±5	123±5
LOD (ng/ml)	3.0	6.0
LOQ (ng/ml)	10.0	20.0

short retention times, hardly no interferences are observed in the chromatogram of drug-free Ringer's solution (Fig. 3a). The signal of diclofenac at concentrations near the LOQ in Ringer's solution (Fig. 3b) and in a Ringer-MD sample (Fig. 3c) offered a good signal-to-noise ratio. Fig. 4 depicts chromatograms of diclofenac in albumin-MD with concentrations below (Fig. 4a) and above (Fig. 4b) the LOQ. The analyte and the internal standard offered a comparable chromatographic behavior with retention times of 3.5 (diclofenac) and of 3.2 min (indomethacine) indicating that the internal standard was well suited to compensate for possible matrix interferences.

A calibration curve was established in the range of 1.02–1047 ng/ml in Ringer's solution with a determination coefficient of $R^2 > 0.9995$. The lower LOQ of 1 ng/ml was validated with a mean accuracy of 92.7% and a precision of 15.5% R.S.D. meeting the requirements for routine analysis [23]. Data concerning the within- and between-day accuracy and precision are summarized in Table 3.

3.3. Comparison of HPLC-UV and HPLC-MS with biological samples

The transdermal penetration was monitored after application of diclofenac gel (Voltaren® Emulgel® 1%) on the thigh of six healthy volunteers. The MD probes were implanted into the subcutaneous adipose tissue directly under the administration site. The probes were perfused either with Ringer's solution or serum albumin (3.3% in Ringer's solution). Samples from the interstitial space fluid were collected in 1 or 2 h intervals up to 6 h. The MD samples were split before analysis and the diclofenac concentrations were determined with two different methods in two different laboratories.

Both analytical methods were evaluated and provided adequate accuracy and sensitivity.

Table 2
Within- and between-day accuracy and precision for the HPLC-UV determination of diclofenac in Ringer's solution

Concentration added (ng/ml)		Concentration found (ng/ml)	Accuracy (%)	Precision (R.S.D.%)
20.0	day 1 ($n=3$)	20.7	103.7	2.7
	day 2 ($n=3$)	21.4	107.2	5.0
	day 3 ($n=3$)	25.3	126.7	2.6
	between-day ($n=9$)	22.5	112.5	3.5
100.0	day 1 ($n=3$)	100.7	100.7	5.8
	day 2 ($n=3$)	94.0	94.0	0.3
	day 3 ($n=3$)	110.9	110.9	0.5
	between-day ($n=9$)	101.9	101.9	2.4
500.0	day 1 ($n=3$)	516.9	103.4	2.3
	day 2 ($n=3$)	541.1	108.2	1.5
	day 3 ($n=3$)	555.9	111.2	1.2
	between-day ($n=9$)	538.0	107.6	1.4

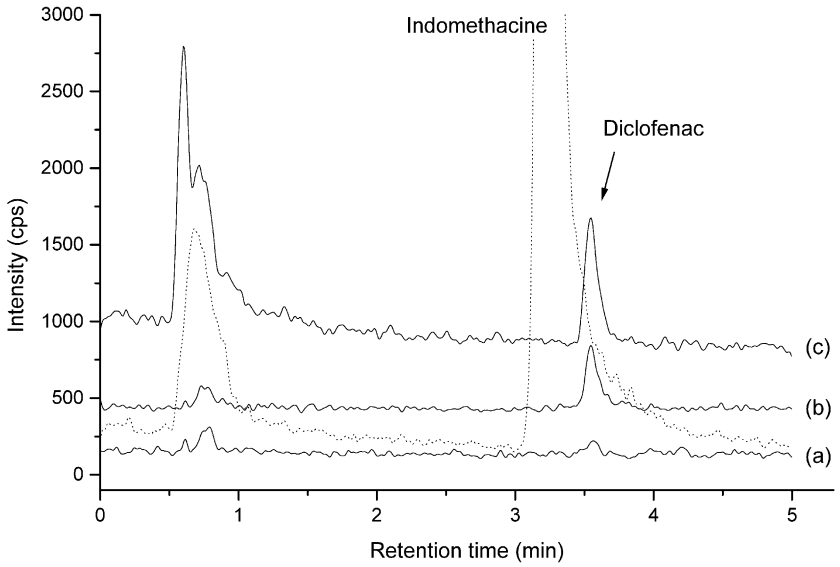

Fig. 3. Diclofenac selective reaction monitoring chromatograms of: (a) a drug-free Ringer's solution; (b) diclofenac standard in Ringer's solution (concentration 1.03 ng/ml); (c) diclofenac in Ringer-MD obtained in vivo from the subcutaneous adipose tissue of a healthy volunteer (calculated concentration 1.84 ng/ml). Recorded fragmentation pathway of diclofenac: m/z 296.2→215.1. Dotted line: Recorded fragmentation pathway m/z 358.2→173.8 of the internal standard indomethacine, concentration 250 ng/ml.

Nevertheless, it became apparent that diverting results were obtained with the two methods when diclofenac was analyzed in biological samples. A comparison of values from selected Ringer-MD samples determined by HPLC-UV and -MS is given in Table 4. It can be seen, that the HPLC-

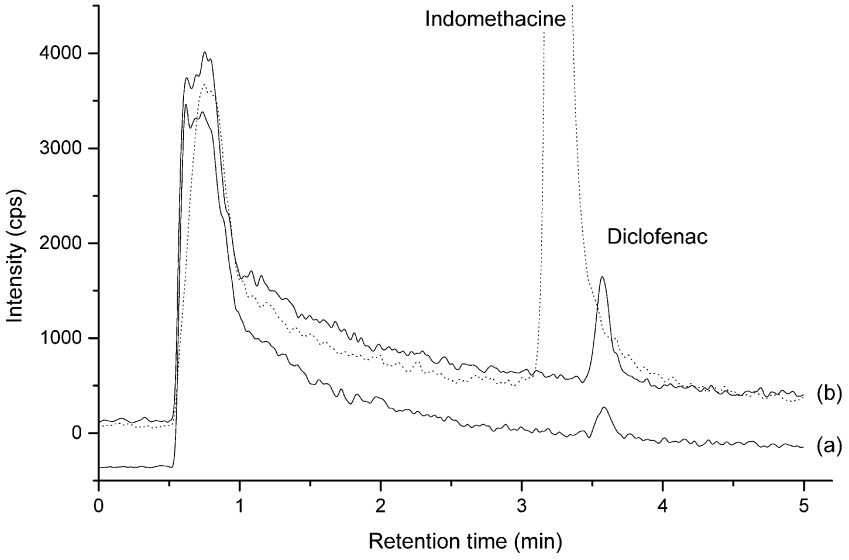

Fig. 4. Diclofenac selective reaction monitoring chromatogram of diclofenac in albumin-MD obtained in vivo from the subcutaneous adipose tissue of a healthy volunteer: (a) concentration below the LOQ; (b) calculated concentration 2.37 ng/ml. Dotted line: trace for internal standard. Experimental conditions see Fig. 3.

Table 3
Within- and between-day accuracy and precision for the HPLC-MS determination of diclofenac in Ringer's solution

Concentration added (ng/ml)		Concentration found (ng/ml)	Accuracy (%)	Precision (R.S.D.%)
2.70	batch 1 ($n = 5$)	2.57	95.2	3.6
	batch 2 ($n = 5$)	2.55	94.4	6.6
	batch 3 ($n = 5$)	2.81	104.1	5.7
	between-batch ($n = 15$)	2.64	97.9	6.9
24.2	batch 1 ($n = 5$)	22.4	92.6	3.9
	batch 2 ($n = 5$)	23.4	96.7	4.9
	batch 3 ($n = 5$)	25.6	105.8	2.3
	between-batch ($n = 15$)	23.8	98.3	6.9
901.6	batch 1 ($n = 5$)	805.5	89.3	3.9
	batch 2 ($n = 5$)	918.6	101.9	1.3
	batch 3 ($n = 4$)	1000.4	110.9	0.37
	between-batch ($n = 14$)	908.2	100.7	9.3

UV assay provided both false positive and negative values. It seemed that some components from the matrix disturbed the assay and generate too high concentration levels. On the other side, there was no reasonable explanation for samples, where too low concentrations were found by UV-detection. Although HPLC-UV offers good accuracy and precision and a low LOD in standard solutions as well as in spiked MD samples, the method failed, when it was applied to biological samples from healthy volunteers. Consequently, HPLC-MS should be preferred for these samples due to its excellent and superior selectivity which is based on the selective mass transition of the parent ions to the product ions. Further advantages of the HPLC-MS method are: (i) only half of the sample

Table 4
Diclofenac concentration in Ringer-MD samples obtained in vivo from two representative healthy volunteers determined by two different methods

Volunteer no.	Time (h)	Diclofenac concentration (ng/ml)		Accuracy of HPLC-UV data (%)[a]
		HPLC-UV	HPLC-MS	
1	0–1	< LOQ	< LOQ[b]	–
	1–2	146.5	164.7	88.9
	2–3	< LOQ	3.22	–
	3–4	101.3	2.42[b]	4020.2
	4–5	< LOD	< LOQ	–
2	0–1	< LOD	< LOQ	–
	1–2	< LOD	< LOQ	–
	2–3	< LOD	36.6	< 5.46
	3–4	< LOD	1.06	–
	4–5	< LOQ	1.42	–
3	0–1	< LOD	< LOQ	–
	1–2	< LOD	< LOQ	–
	2–3	< LOD	< LOQ	–
	3–4	< LOQ	4.23	–
	4–5	15.4	< LOQ[b]	> 1500

[a] HPLC-MS data were set to an accuracy of 100%.
[b] Two independent determinations.

volume was required allowing to reanalyze the samples; (ii) superior accuracy due to the use of an internal standard; (iii) strikingly reduced run times which enabled higher sample through-puts.

3.4. Clinical application of the assay

In all Ringer-MD samples only low diclofenac concentrations could be detected by both HPLC-UV and -MS. Despite the high sensitivity of the assays the diclofenac concentration of most of the samples were under the LOQ, e.g. volunteer no. 3 in Table 4. A possible explanation for these unexpected low concentration levels is that only the free form of a drug diffuses from the interstitial space fluid through the MD membrane into the perfusate. Due to its high affinity to proteins of 99.5% diclofenac may be bound to interstitial space or cell membrane components, and is consequently non-accessible to Ringer-MD experiments.

To increase the analyte concentration in MD samples, a new approach was taken. Serum albumin was added to the perfusate to shift the equilibrium of diclofenac bound to extracellular components to serum albumin in the perfusate. In fact distinctly higher diclofenac concentrations were found in several albumin-MD samples, although the concentration of some samples still lay under the LOQ. Therefore, this approach was only partly suitable to increase the recovery of the MD experiment. Detailed results of the clinical study will be published elsewhere (manuscript in preparation).

4. Conclusions

Two reversed-phase HPLC methods based on UV or tandem MS detection were developed and evaluated in two different laboratories. The methods were applied to the same MD samples and the results were compared. The HPLC-UV detection failed to quantify diclofenac in biological samples as both false negative and false positive data were found. MD samples seemed to contain matrix components which disturb the assay, although no interferences for diclofenac standards in Ringer's solution and in spiked MD samples were found during evaluation of the assay. Therefore, the use of the HPLC-MS method is highly recommended for biological samples, as the detection is much more selective. Furthermore, the HPLC-MS method is superior to the HPLC-UV assay in terms of sensitivity and time needed for sample preparation and separation.

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